DEFENSIVE SYNERGY: THE ANTIPREDATORY ROLE OF GLASS SPICULES IN CARIBBEAN DEMOSPONGES

Adam C. Jones

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Approved by

Advisory Committee

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Chair

Accepted by

_____
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ABSTRACT

Many sponge species produce secondary metabolites that deter predation. Sponges also contain siliceous spicules, but previous studies have provided little evidence that spicules offer any defense against generalist fish predators. However, feeding assays in past investigations tested sponge skeletal elements in isolation, and did not consider whether they may enhance chemical defenses. To determine whether the two components have an additive, antagonistic, or synergistic interaction, crude organic extracts and spicules from 8 Caribbean sponge species were isolated and tested in laboratory assays. The sponges used included one chemically defended reef sponge (*Agelas clathrodes*) and seven sponges known to be intermediately deterrent. These included six reef species (*Calyx podotypa*, *Cinachyra alloclada*, *Cribrochalina vasculum*, *Niphates digitalis*, *Rhaphidophlus juniperinus*, and *Xestospongia muta*) and one mangrove species (*Tedania ignis*). Extracts and spicules were used in various concentrations, both individually and in combination, in laboratory feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*. A SAS based GENMOD procedure based on an isobolographic analysis model was used for statistical comparisons. Four sponges (*A. clathrodes*, *C. alloclada*, *R. juniperinus*, and *X. muta*) showed evidence of synergisms. Of these species, synergy in *C. alloclada*, *R. juniperinus*, and *X. muta* was caused by approximate natural concentrations of extracts and spicules. Extracts of *A. clathrodes* were deterrent, but combination assays required unnatural reductions in extract concentrations and increases in spicule concentrations to show the synergistic effect. Contrary to previous findings, spicules from *Cribrochalina vasculum* and *X. muta* were deterrent at natural concentrations. These results indicate that, for some sponges, structural elements may serve to enhance chemical defenses against consumers.

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INTRODUCTION

Sessile organisms that employ defensive mechanisms are faced with the dilemma of allocating enough energy for competitive development and reproduction, but at the same time successfully defending against predation, pathogens, and overgrowth (Levin 1976; Herms and Mattson 1992). Consequently, fitness losses must be minimized through escape, tolerance, or deterrence (Lubchenco and Gaines 1981). Structural defenses against predation employed by terrestrial and marine plant species include the use of spines (e.g. Cooper and Owen-Smith 1986), thorns and resin ducts (e.g. Maxwell et al. 1972; Hanover 1975), tissue toughness (e.g. Howard 1988; Pennings and Paul 1992) or reductions in nutritional quality available to consumers (Duffy and Paul 1992). However, plant secondary metabolites, i.e., those compounds not involved in primary metabolic functions (Berenbaum and Neal 1985) may be the most important mechanism of defense against herbivory (e.g. Howard 1988; Schultz 1988). Secondary metabolites are diverse (e.g. Faulkner 2000, and previous citations therein) and are also prevalent in terrestrial and marine invertebrates, particularly in marine tropical zones (e.g. Bakus and Green 1974; Pawlik 1993; Steinberg et al. 1995; Bolser and Hay 1996). On coral reefs, secondary metabolites appear to be most common among benthic organisms that are vulnerable to high predation rates (e.g. Hay and Steinberg 1992; Paul 1992; Pawlik 1993; Hay 1996b). These organisms include sponges, soft corals, sea slugs, and tunicates, which all lack obvious physical means of deterring predation by consumers (Pawlik 1993).

Sponges are important constituents of reef ecosystems and are among the most abundant and diverse groups of sessile invertebrates in the Caribbean (e.g. Wiedenmayer 1977; Suchanek et al. 1983; Alcolado 1991; Alvarez et al. 1991a, b; Schmahl 1991; Wulff 1997). Sponges are a rich protein source (Chanas and Pawlik 1995) and the soft, fleshy tissue of many sponges

appears to make them targets in areas noted for high predation rates (Hixon 1983, Carpenter 1986). However, relatively few spongivores have been identified, including some fishes (Randall and Hartman 1968; Wulff 1994, 1997; Dunlap and Pawlik 1996), hawksbill turtles (Meylan 1988) and invertebrates such as opisthobranchs (Pawlik et al. 1988) and echinoids (Birenheide 1993).

While sponges are soft-bodied organisms, many produce structural components as part of their skeleton. Structural components include spicules that are either siliceous (e.g. Schönberg 2001) or calcareous (e.g. Bidder 1898-1899, Ilan et al. 1996) and proteinaceous spongin fibers (Bergquist 1978; Chanas and Pawlik 1996). Siliceous spicules are formed in cells called sclerocytes, in which amorphous silica is secreted in layers around an organic axial thread (Levi 1963; Weissenfels and Landschoff 1977). Koehl (1982) described how sponge spicules strengthen the sponge skeleton by increasing tissue rigidity. A positive correlation between sponge spicule density and flow regimes has also been demonstrated (e.g. Palumbi 1986; McDonald et al. 2002), in which spicule content is increased as a form of structural support against elevations in wave force. However, Bell et al. (2002) found that spicules in *Cliona celata* became longer, narrower, and less numerous in high energy sites, possibly as a way of making the sponge more flexible. Uriz et al. (1995) observed that the Mediterranean encrusting sponge *Crambe crambe* has an increased spicule density and amount of collagen in shaded areas, and a smaller investment in growth and reproduction than other *C. crambe* sponges found in illuminated areas.

Sponges provide the greatest diversity of marine natural products (Braekman et al. 1989; Faulkner 2000, and previous citations therein). Sponge secondary metabolites are complex molecules, can be present in high concentrations (Rogers and Paul 1991) and are thought to have

high-energy construction costs. Classes of secondary metabolites include sterols, terpenoids, amino acid derivatives, saponins, and macrolides (Sarma et al.1993; Wilson et al. 1999). These compounds have been studied for their pharmacological applications (e.g. Kitagawa and Kobayashi 1993; Flam 1994; Munro et al. 1994), as well as for their ecological functions. Secondary metabolites from several sponge species are involved in allelopathic spatial competition (e.g. Sullivan et al. 1983; Engel and Pawlik 2000), inhibition of bacterial colonization and fouling (e.g. Newbold et al. 1999; Kelly et al. 2003), and protection from ultraviolet radiation (Paul 1992). However, more attention has been devoted to studying how these metabolites may offer protection against potential predators (e.g. Pawlik et al. 1995; Uriz et al. 1996; Pawlik 1997).

Crude organic extracts from many sponges deter feeding by generalist fish predators and invertebrates. Pawlik et al. (1995) found that of 71 Caribbean sponges, 69% yielded extracts that when incorporated into artificial foods at natural volumetric concentrations were deterrent in aquarium assays with the bluehead wrasse, *Thalassoma bifasciatum*. In two separate studies, Waddell and Pawlik (2000a, b) demonstrated that crude extracts from several sponge species deterred feeding by the hermit crab *Paguristes punticeps* and the sea stars *Echinaster echinophorus* and *Echinaster sentus*.

Levels of deterrence offered by sponge extracts can also vary among individuals of the same species. Chanas and Pawlik (1997) observed that 60 specimens of the barrel sponge *Xestospongia muta* yielded crude organic extracts that ranged in levels of palatability based on geographic location, and Swearingen and Pawlik (1998) noted significant variability in deterrence in the sponge *Chondrilla nucula*, based on geographic location and month of sample collection.

The defensive role of physical structures in reef invertebrates is controversial. Harvell et al. (1988) found that purified calcitic sclerites from the gorgonian *Pseudopterogorgia acerosa* inhibit feeding, but O'Neal and Pawlik (2002) tested natural volumetric concentrations of sclerites from 32 Caribbean gorgonian species and discovered that 30 of the species (including *P. acerosa*) were palatable in aquarium assays with *T. bifasciatum*. Sponge spicules have been found in gut and intestinal content analyses of those spongivores listed previously, and Chanas and Pawlik (1995) found that spicules from eight demosponge species did not deter feeding in laboratory assays with *T.bifasciatum*, or in field assays with a natural assemblage of reef fish. Conversely, Burns and Ilan (2003) did find that spicules from 4 Caribbean sponges and 2 Red Sea sponges deterred feeding with the Red Sea wrasse *Thalassoma klunzingeri*, and that assays combining natural concentrations of crude organic extract and spicules from the sponge *Crella cyatophora* were significantly more deterrent than either the extract or spicules by themselves. Hill and Hill (2002) found that spicule density increased in response to artificial sponge tissue damage, and proposed that structural elements may function as inducible anti-predator defenses. However, the sponge tissue from which Hill and Hill obtained spicule density measurements was repeatedly wounded and was probably not comparable to new tissue formed after a predation event. Hill and Hill also did not provide feeding assay data to suggest that increased spicule density coincides with decreased predation. The nature of the relationship between secondary metabolites and spicules in sponges has yet to be systematically explored. If sponges follow similar tradeoffs between growth and defense as those proposed in plants (Fagerstrom 1989) evidence of interactions in sponges that elevate defense levels could imply that less energy in sponges is being expended to secondary metabolite construction, and would allow for faster growth rates.

Interactions between physical or chemical agents can be defined as additive, antagonistic, or synergistic, depending on the net effect resulting from their combination. The nature of the interaction is best described algebraically. If, for example, two agents of respective potencies (Z_1*, Z_2*) are combined to achieve a certain effect, this effect is defined as additive if (Z_1/Z_1*) + (Z_2/Z_2*) = 1, where Z refers to the amount of each unit to be added. Conversely, antagonistic agents would yield a value less than 1, while synergistic agents are greater than 1 (Tallarida 1992). Therefore, synergism is "the joint action of different substances in producing an effect greater than the sum of the effects of all substances acting separately" (Funk and Wagnalls 1968) and exists in any case in which one compound elevates the biological activity of another (Wilkinson 1973) such as when one defense makes another more potent (Pennings 1996). A combination is considered additive if the joint action of different substances does not differ from the sum effect of each substance, meaning that no interaction is present. An antagonistic interaction occurs when a combination of substances decreases the overall effect in relation to the sum effect of each substance (Berenbaum 1988).

Synergism and antagonism have been addressed widely in pharmaceutical literature, but have received less attention in terrestrial and marine chemical ecology. Recent pharmaceutical research has examined synergism in drug interactions using rats, including studies of pain relief (e.g. Graham et al. 2000; Han et al. 2000; Shannon et al. 2001) weight loss (Rowland et al. 2000) and damage caused by illicit drugs (Tallarida et al. 1997a). In terrestrial chemical ecology, Berenbaum and Neal (1985) found that myristicin, a methylenedioxyphenyl containing phenylpropene compound in plants of the family Umbelliferae, synergistically interacts with the furanocoumarin xanthotoxin to elevate toxicity against the corn earworm *Heliothis zea*. However, Diawara et al. (1993) showed that combinations of xanthotoxin had an antagonistic

relationship with two other furanocoumarins, psoralen or bergapten, significantly reducing their toxicity towards the beet armyworm *Spodoptera exigua*. Frankmölle et al. (1992) found that laxaphycins from the terrestrial blue-green alga *Anabaena laxa* synergistically interacted to inhibit fungal growth in an agar diffusion assay. In marine systems, Hay et al. (1994) detected synergism when semipurified secondary metabolites from the calcified green seaweeds *Rhipocephalus phoenix* or *Udotea cyathiformis* were combined with finely powdered calcium carbonate ($CaCO_3$). The findings of Hay et al. (1994) were subject to some debate: Pennings (1996) argued that a multiplicative null hypothesis was more appropriate than the additive null hypothesis used by Hay et al. (1994) and that synergisms may have been incorrectly defined, but Hay (1996a) responded by showing limitations identified by Pennings were not relevant to the Hay et al. (1994) study, and that synergy was present.

Statistical approaches to analyze data for evidence of interactions have varied depending on the field of study. Isobolographic analysis (Fraser 1870-1871; Loewe and Muischnek 1926) is a common method for examining interactions in pharmacology and pathology (e.g. Berenbaum 1988; Gessner 1988; Tallarida 1992; Tallarida et al. 1997b). In an isobolographic analysis, the composition of dose mixtures of two agents are expressed graphically using rectangular coordinates, with the doses being defined by reference to the abscissa and ordinate (Gessner 1988). A line connects the intercepts of Z_1* and Z_2*, which represents all of the possible isoeffective combinations. A second, radial line denotes all possible coordinates of the chosen proportions for the experimental mixture (Z_{mix}), and the intersection of these two lines has the coordinates [(Z_1/Z_1*), (Z_2/Z_2*)]. The location of the coordinates from Z_{mix} determines the overall effect of the combination. If the coordinates are not significantly different from the line, the mixture is additive; if below or above the line, the mixture is synergistic or antagonistic,

respectively. Confidence intervals for the line can be constructed, and variance between coordinates can be statistically analyzed (Tallarida et al. 1997b; Nelson and Kursar 1999).

Other statistical models for comparing interactions, including ANOVA, the Case and Bender test (1981), and multiplicative tests (Wootton 1994) are usually targeted at interactions between species but as Hay (1996b) notes may also be applicable to situations involving potential synergisms. Billick and Case (1994) state that intended comparisons using these models may not be achieved because statistical analyses can be difficult to implement properly, and be applied to interactions inappropriately assumed to be relevant. There are several characteristics of the isobolographic model that are preferable to such tests as ANOVAs when analyzing dose-response data. No assumptions are made about the interaction, and data do not have to be linear or limited to a normal distribution (Nelson and Kursar 1999). The interaction between components can also be easily depicted and interpreted using the graphic isobole. The isobole allows for identification of maximum synergy or antagonism, and to isolate specific combinations that may yield anomalous effects (Berenbaum 1977). Only one attempt to apply isobolographic analysis to ecological data has been made (Nelson and Kursar 1999) in which the results of Berenbaum and Neal (1985) were verified. The use of both isobolographic analysis and statistical models employing the isobole format can serve as valuable tools in ecology studies.

The use of statistical methods for categorical data analysis (CDA) has become increasingly important in recent years, especially in the biomedical and social sciences. Categorical data is comprised of variables for which the measurement scale is a set of categories (Agresti 1996). Fisher and Yates (1938) proposed log [л/(1- л)] as a binomial parameter transformation for binary data analysis. Joseph Berkson (1944) later called this a “logit”

transformation. An effective method of comparing effects of explanatory variables on categorical response variables is the generalized linear model (GENMOD). All GENMODs have three components: the random component, the systematic component, and the link. The random component identifies a response variable Y and assumes a particular distribution for it. The systematic component identifies the explanatory variables, which are used as model predictors. The link is the functional relationship between the mean of the random component and the systematic component. The GENMOD uses a linear prediction equation to relate a function of the mean to the explanatory variables (Agresti 1996). A GENMOD with a normal distribution is appropriate for tests such as ANOVAs, while a binomial distribution using a logit transformation would be useful for examining probability of success or failure in a set number of trials. The binomial distribution could be applied to ecological assays to predict predator responses to food items in varying concentrations. In this study, a SAS based GENMOD procedure modeled on isobolographic analysis was used to consider the relationship between secondary metabolites and spicules in sponges collected in the Florida Keys and Bahamas. Because spicules in isolation have, in most cases, no deterrent effect against predation, I examined whether the addition of spicules to crude organic extracts containing secondary metabolites resulted in an interaction that changed the level of deterrency of these metabolites in aquarium feeding assays using the bluehead wrasse, *Thalassoma bifasciatum*.

MATERIALS AND METHODS

Sponge identification and collection

Sponges for this study were collected from Jewfish Creek (25 º11.24N, 80 º23.27W), North North Dry Rocks (25º07.850N, 80º17.521W) and Pickles Reef (24 º59.07 N 80º24.97 W), Florida Keys, and San Salvador (24º03.095N, 74º32.372W) and South Abaco (77º29 W, 25º98 N) Bahamas at depths of 10-30 meters using SCUBA. The sponge species used were based on feeding assay data reported by Pawlik et al. (1995): one chemically defended species (*Agelas clathrodes*) and seven species shown to have intermediate levels of defense: (*Calyx podotypa, Cinachyra alloclada*, *Cribrochalina vasculum*, *Niphates digitalis*, *Rhaphidophlus juniperinus*, *Tedania ignis,* and *Xestospongia muta*). *Tedania ignis* was collected in mangrove habitats, while all other sponges were found on reefs. Although one individual of each species was used in all procedures, two additional *X. muta* sponge samples were used that were both collected at South Abaco, Bahamas, but had notably different morphologies. Portions of sponges were obtained either by gentle tearing or cutting tissue with a sharp knife, and were frozen at -20° C until used for extractions and spicule collection.

Crude organic extract isolation

Crude organic extracts containing secondary metabolites from sponges were isolated based on procedures described in Pawlik et al. (1995). Sponge portions were chopped into 1x1 cm pieces and 200 mL of sponge tissue was measured using volumetric displacement of water in a graduated cylinder. The water was drained and the tissue was moved to a 1L Nalgene bottle, where it was extracted in 1:1 dichloromethane:methanol (DCM:MeOH) for 24 hours. During this time, water combined with the methanol and the MeOH:water phase separated from the

dichloromethane phase. These phases were partitioned using a separatory funnel, vacuum filtered through celite, and the MeOH:water phase was evaporated to dryness in a round bottom flask using low heat (<40°C) on a rotary evaporator. The sponge tissue was then extracted again in methanol for 24 hours. The methanol extract was added to the dried MeOH:water extract and dried down to completion. Following this, the DCM partition was added to the round bottom flask and evaporated. This combination was then resuspended and moved in 10 mL equivalents into 20 mL scintillation vials, where remaining residual solvents were removed by rotary evaporation and vacuum concentration.

Spicule isolation

Spicules from each sponge were treated using methods based on Chanas and Pawlik (1995). In order to determine an average amount of spicule content per mL of sponge tissue, each 200 mL equivalent of tissue was placed in an inverted 2 L soft drink bottle after extraction. The bottom of the bottle was removed and the opening was outfitted with a valve. Chlorine bleach (sodium hypochlorite, 6%) was added to the tissue, and as the tissue oxidized spicules collected at the mouth of the bottle. Several bleach additions were usually needed to oxidize all of the tissue. After the bleach had stopped bubbling the spicules that collected in the bottle were drained through the valve through 52 μm mesh. The material collected on the mesh was removed and allowed to dry on wax paper. The filtrate was collected and filtered again through a 5 μm polycarbonate Millepore isopore membrane filter using acetone rinses to obtain any remaining spicules. All spicules were rinsed again in bleach to dissolve any remaining tissue and moved to a plastic centrifuge tube. The bleach was then removed using a pipet, and the pellet was rinsed three times in distilled water. The pellet was then treated with a 1M solution of

sodium thiosulfate for approximately 15 minutes to neutralize any remaining bleach. The pellet was rinsed twice more in distilled water, and then suspended in a small amount of acetone. The solution was filtered a final time through another 5 μm filter, and the pellet was broken up into small pieces and allowed to dry. The mass of the pellet was obtained for each sponge, and spicule content was standardized to 200 mL of tissue.

Feeding assays

The use of the bluehead wrasse, *Thalassoma bifasciatum*, in aquarium bioassays has been detailed previously (Pawlik et al. 1987). It is one of the most abundant fish on Caribbean reefs, and is a generalist carnivore that samples several different invertebrate species (Randall 1967). Generalist predators were used in this study because generalists would be less likely than specialists to have evolved ways of avoiding defenses, and invertebrate defense mechanisms would be directed against them in particular because of their high numbers. Bioassays used in the present study are based on the methodology described by Pawlik et al. (1995).

To each vial containing crude extract from the original 200 mL extraction, a composite matrix of 5 g freeze dried, powdered squid mantle, 3 g high viscosity, sodium salt alginic acid, and 100 mL water was added using a graduated syringe. The volumetric amounts of crude organic extract and squid matrix were varied to create different concentrations; for example, 2.5 mL of squid matrix added to a 2.5 mL crude extract equivalent would constitute a natural concentration of metabolites, while 5 mL of squid matrix added to a 2.5 mL crude extract equivalent would create a concentration one-half that found in the sponge. The mixture was vigorously stirred with a metal spatula to homogenize and suspend the extract into the matrix. The matrix was viscous enough to prevent any settling of assay ingredients. A control mixture

was prepared in the same way, but without the addition of the crude extract. Both the control and treatment mixtures are treated with minimal amounts of food coloring until they matched in color. Each mixture was then separately loaded into a 3 mL syringe, and extruded into a .25 M solution of calcium chloride to form a long, spaghetti-like strand. After a few minutes, the hardened strands were removed, and chopped into 4 mm long pieces with a razor blade. Food pellets containing spicules were created using weighed spicule amounts proportional to the volumetric densities established using the bleaching process. Squid matrix was extruded into the vial containing the spicules, and the contents were gently stirred to homogeneously distribute the spicules while avoiding breakage. On occasion, sample pellets were bleached again and spicules were examined for any damage incurred during mixing.

Control and treated pellets were presented to groups of 2 to 5 yellow or blue phase bluehead wrasses, *Thalassoma bifasciatum*, that had been divided randomly into multiple cells among 30 gallon aquaria. For each cell in the assay, the fish were given a control pellet. If the control pellet was consumed, a treatment pellet containing either secondary metabolites or spicules was offered. If the fish rejected the treatment pellet, another control pellet was given to determine if the fish had ceased to feed. A pellet was considered rejected if the pellet was not eaten (engulfed and spit out) after a total of three attempts by one or more of the fish in that particular group. Fish that did not eat control pellets were not used, and at least 10 fish cells were required for each individual assay. Feeding assays were conducted until concentrations of crude extract and spicules were found that yielded intermediate levels of deterrency (or ED_{50}, for 50% effective dose). In practice, ED_{50} concentrations used in assays ranged from 3 to 7 pellets out of 10 eaten.

A series of additional, combination assays using both crude extract and spicules in a range of concentrations were then performed. All combination assays used concentrations of each ingredient that were equal to or less than the ED_{50} values. For preparation of the food pellets, the squid matrix was added to a dried extract sample and vigorously mixed. Food coloring was then added, and spicules were added last and stirred gently to avoid breakage. In each assay, the ratio of squid matrix added to both the extract and spicules was designed to create different concentrations of each ingredient. Before conducting the combination assays, assays with pellets containing ED_{50} concentrations of either crude extract or spicules were conducted to verify that the assay fish being used were not starving or satiated. When possible, combination assays were run multiple times using different sets of 10 fish cells. Combination assays for *C. alloclada* run 2 times composed 16% of the total assay data for the sponge. For *A. clathrodes*, *C. podotypa*, and *C. vasculum*, combination assays run 2 or more times composed at least 40% of each data set, and for all other sponges, all multiple runs composed at least 50% of each data set.

Statistical procedures

A SAS (version 8, 2001, SAS Institute, Cary, NC) based generalized linear model (GENMOD) procedure was used to analyze data collected from all feeding assays conducted (for an extensive review of generalized linear procedures, see Agresti 1996; abbreviated explanations given here are based on explanations from this text). A "cards" input design was used in SAS. Using this method, the concentration of each predictor (crude extract or spicules) could be given relative to natural concentration, followed by the number of pellets accepted out of total number of trials. In cases where more than one replicate of the same assay concentration was conducted, each replicate was entered as a separate data point. Assays using only crude extract, only

spicules, combinations of crude extract and spicules, or with neither included (control pellets; always 10/10 accepted) were entered into the data set for each sponge.

For the design of the generalized linear model, a binomial probability distribution was selected, which is appropriate when each response variable Y_i is the number of "successes" out of a certain fixed number of trials (Agresti 1996). For each sponge, $\beta_1 E$, $\beta_2 S$, and $\beta_3 ES$ respectively referred to data value estimates for an extract assay, a spicule assay, and a combination assay (where E and S are variables corresponding to extract and spicules), and β_0 identified the intercept. The link function for the model was a logit link, defined as $g(\pi) = \log[\pi /(1- \pi)]$. Using the link, the null model is:

$$\log[\pi(Y) /(1- \pi(Y))] = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 E S \qquad \text{(equation 1)}$$

Equation 1 can also be rearranged in terms of probability:

$$\pi(Y): = e^{(\beta_0 + \beta_1 E + \beta_2 S + \beta_3 E S) / (1+e^{(\beta 0 + \beta_1 E + \beta_2 S + \beta_3 E S))} \quad \text{(equation 2)}$$

Analysis of parameter estimates for each sponge was supplied by the SAS GENMOD procedure. Deviance for each model was compared to a χ^2 distribution to determine goodness of fit. Values for β_0, βE, βS, and βES were obtained from these estimates. The feeding assay data from each sponge was used to create 3-D and 2-D plots that resembled isobole examples as provided in several sources (e.g. Greco et al. 1995; Tallarida et al. 1997b; Nelson and Kursar 1999). For the 3-D plots, equation 2 was graphed with the β values included for each sponge. For the 2-D plots, the null model was solved for ED_{50}, which eliminates the log function on the left side of the equation (since log 1 = 0):

$$\mu = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 E S \quad \text{(equation 3)}$$

The null model can also be rearranged to examine the effect of spicules:

$$S = (\beta_1 E + \beta_0)/- (\beta_2 + \beta_3 E) \qquad \text{(equation 4)}$$

Equation 4 was graphed on the 2-D plots with the extract-spicule interaction both included and excluded.

The X and Y intercepts on the 3-D plot represented the ED_{50} values for crude extract and spicule concentrations as determined by the model. A plane intersecting the ED_{50} values was also included as a reference. 2-D plots were created to obtain an additional view of the function/plane interface from the 3-D plot. Each 2-D plot included the interaction function (IF), the additive line connecting the model-derived ED_{50} values (MA), and another additive line connecting the ED_{50} values determined from assay results (EA).

The type of relationship between crude extract and spicules for each sponge was assessed using the results of the GENMOD procedure, and the shape of the function curve in relation to the additive line. An additive interaction was noted if the probability of acceptance was not significantly different than that predicted by the χ^2 distribution. A synergistic or antagonistic interaction was observed if the probability of acceptance was significantly lower or higher than that predicted by the χ^2 distribution, respectively. A plot of a synergistic interaction would create a concave curve below the additive line, and a plot of an antagonistic interaction would create a convex curve above the additive line. If synergisms were present, comparisons were also made to determine the difference in the ED_{50} value of crude extract alone and the ED_{50} value of crude extract combined with a natural concentration of spicules.

RESULTS

Volumetrically derived spicule densities for each of the ten sponge samples used in this study are given in table 1. Synergistic interactions between crude extracts and spicules were noted in four species, *Agelas clathrodes, Cinachyra alloclada, Rhaphidophlus juniperinus,* and one sample of *Xestospongia muta* (Figures 1-10). In most cases, ED_{50} values for crude extract and spicules for each sponge found by conducting assays were similar to those predicted by the model. Comparisons between combination assays and those assays using only crude extract and spicules were made using the IF and MA plots on the 2-D graphs. There did not appear to be a noteworthy amount of spicule breakage in those assay pellets that were bleached for inspection.

The crude extract of *Agelas clathrodes* at natural concentration exhibited the highest level of deterrence of all sponges tested. This was in accordance with crude extract assays conducted by Pawlik et al. (1995), who observed a mean of 0 out of 10 pellets consumed at natural concentration. The plots show that the ED_{50} for crude extract was approximately 0.37X while the ED_{50} for spicules was approximately 8.6X. A synergistic interaction between crude extract and spicules was noted (GENMOD, $p < 0.01$; Figure 1).

The ED_{50} values for *Cinachyra alloclada* were approximately 0.40X crude extract and 1.7X spicules. Synergism was also noted in this case (GENMOD, $p = 0.02$; Figure 3). According to the 2-D plot, when combined with 1X spicules, only 0.06X crude extract is required to create an ED_{50}. The crude extract ED_{50} value for *Rhaphidophlus juniperinus* was close to natural concentrations (1.1X), while the spicule ED_{50} was 5.5X.

Table 1: Spicule density from each volumetric (200 mL) sponge sample.

Sponge	spicule density g/mL
Agelas clathrodes	0.0052
Calyx podotypa	0.0116
Cinachyra alloclada	0.0276
Cribrochalina vasculum	0.0294
Niphates digitalis	0.0094
Rhaphodophlus juniperinus	0.0102
Tedania ignis	0.0146
Xestospongia muta Florida Keys sample	0.0397
Xestospongia muta Bahamas,hard morphology	0.0692
Xestospongia muta Bahamas, soft morphology	0.024

Table 2: Comparison of experimental and model derived ED_{50} values for extract and spicule concentrations, total number of assays conducted per sponge, and deviance from χ^2 distribution for goodness of fit for each model, where null model = 1.

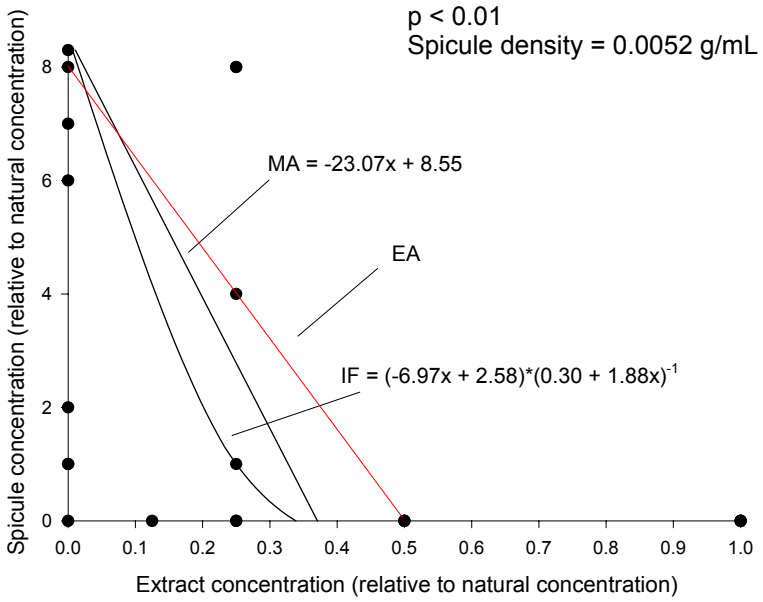
Sponge	Experimental ED_{50} [Extract]	Model ED_{50} [Extract]	Experimental ED_{50} [Spicule]	Model ED_{50} [Spicule]	Total Assays	Model Deviance
Agelas clathrodes	0.5X	0.37X	8X	8.6X	22	1.34
Calyx podotypa	2X	1.9X	2X	2.0X	25	2.77
Cinachyra alloclada	0.5X	0.40X	2X	1.7X	19	2.11
Cribrochalina vasculum	4X	4.0X	1X	0.91X	16	2.33
Niphates digitalis	1X	2.9X	2X	2.7X	23	0.925
Rhaphidophlus juniperinus	1X	1.1X	4X	5.5X	25	1.77
Tedania ignis	2X	2.2X	1X	1.3X	17	2.52
Xestospongia muta, Florida Keys sample	1X	0.80X	1X	0.93X	17	1.27
Xestospongia muta, Bahamas hard morphology	1X	1.1X	1X	1.1X	23	2.52
Xestospongia muta, Bahamas soft morphology	1X	0.75X	4X	4.1X	28	1.45

The graphs show that a combination of a natural concentration of spicules with a reduced extract concentration (0.4X, less than half that concentration needed in isolation) was also an ED_{50}. This interaction was synergistic (GENMOD, $p < 0.01$; Figure 6).

Additive interactions (no significant relationship) between crude extract and spicules were found for *Calyx podotypa* (GENMOD, $P = 0.66$; Figure 2), *Cribrochalina vasculum* (GENMOD, $p = 0.18$; Figure 4), *Niphates digitalis* (GENMOD, $p = 0.92$; Figure 5), and *Tedania ignis* (GENMOD, $p = 0.84$; Figure 7).

Results varied considerably among the three samples of *Xestospongia muta*. The structural morphology of each individual appeared in the field to be different: one sample collected at South Abaco, Bahamas was rigid and required cutting with a sharp knife to obtain it, while another about 5 m away was soft and crumbled when touched. The third sample, collected in the Florida Keys, resembled the more rigid sponge found in the Bahamas. All of the *X. muta* sponges appeared to be healthy and did not exhibit signs of bleaching. The spicule densities varied (Table 1). The ED_{50} values for the crude extracts were similar (Bahamas soft *X. muta* = 0.76X, Bahamas hard *X. muta* = 1.1X, Keys *X. muta* = 0.80X) while the spicule concentration required by the Bahamas soft *X. muta* was higher (4.1X) than the Bahamas hard *X. muta* (1.6X) or Keys *X. muta* (0.9X). However, only the Keys *X. muta* was synergistic (GENMOD, $p < 0.01$; Figure 8) while the others (Bahamas hard *X. muta*: GENMOD, $p = 0.47$; Figure 9; Bahamas soft *X. muta*: GENMOD, $p = 0.91$, Figure 10) were additive. Approximately 0.25X concentrations of crude extract and spicules in combination were required in the Florida Keys *X. muta* sample to reach the ED_{50} level.

a)



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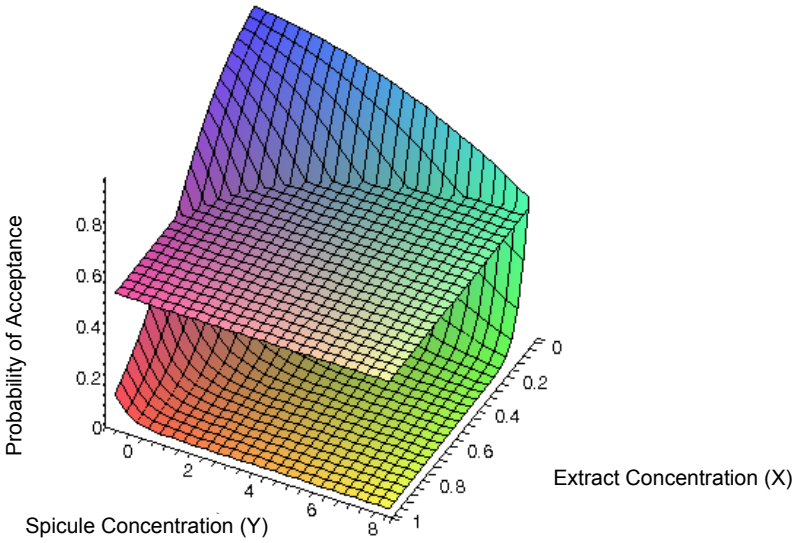
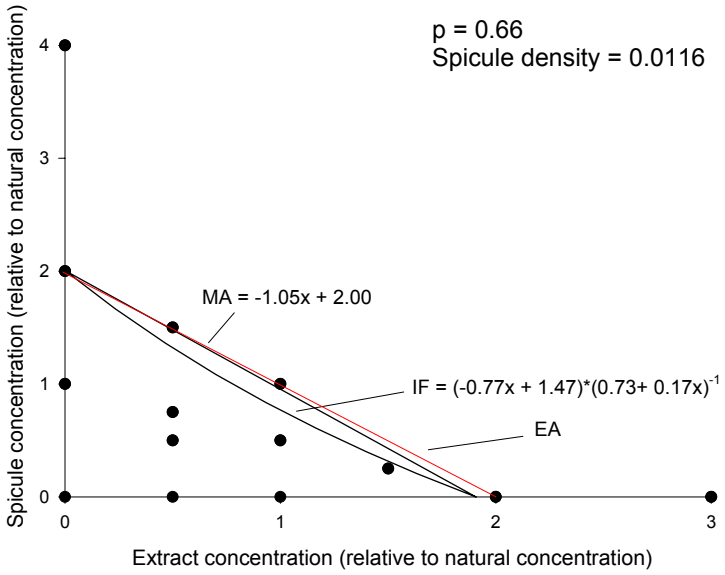


Figure 1: a) 2-D Isobologram for *Agelas clathrodes*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Agelas clathrodes*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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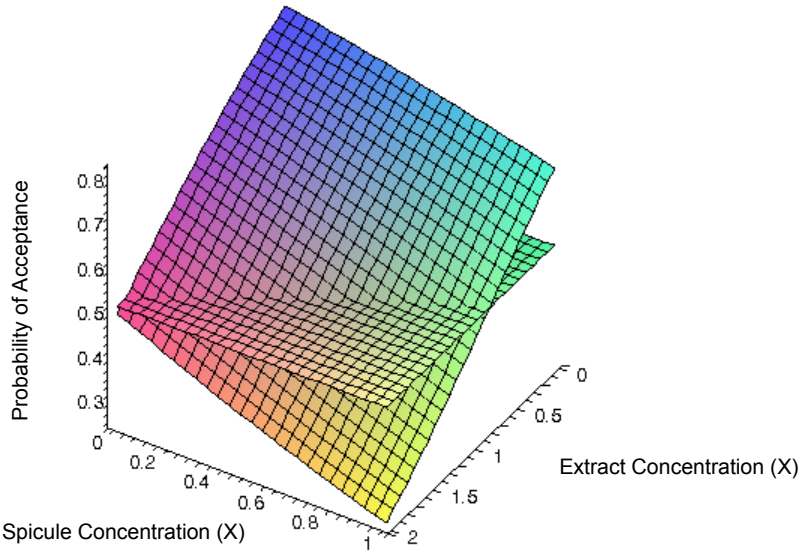
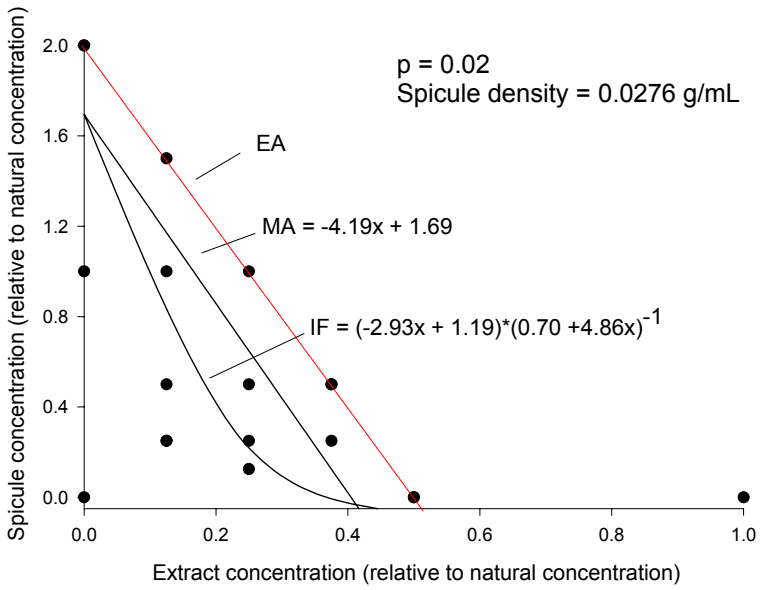


Figure 2: a) 2-D Isobologram for *Calyx podotypa*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Calyx podotypa*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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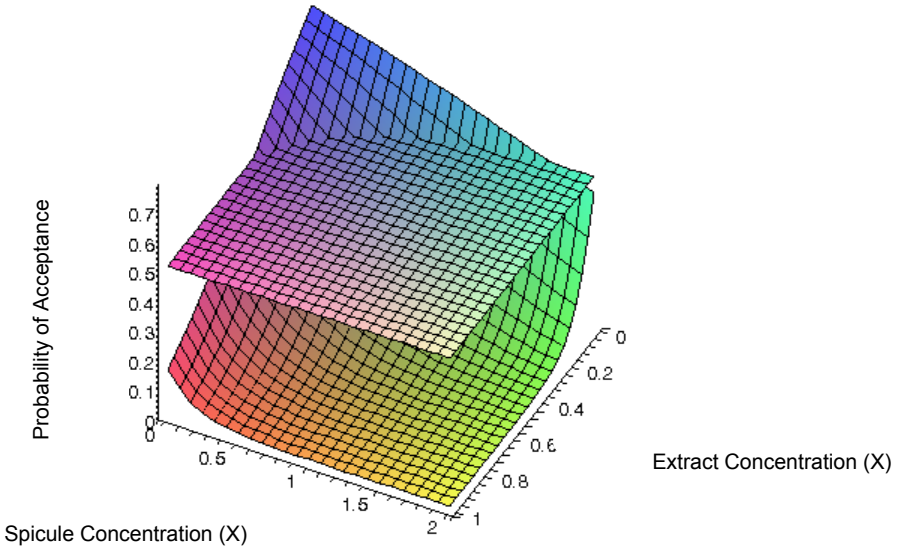
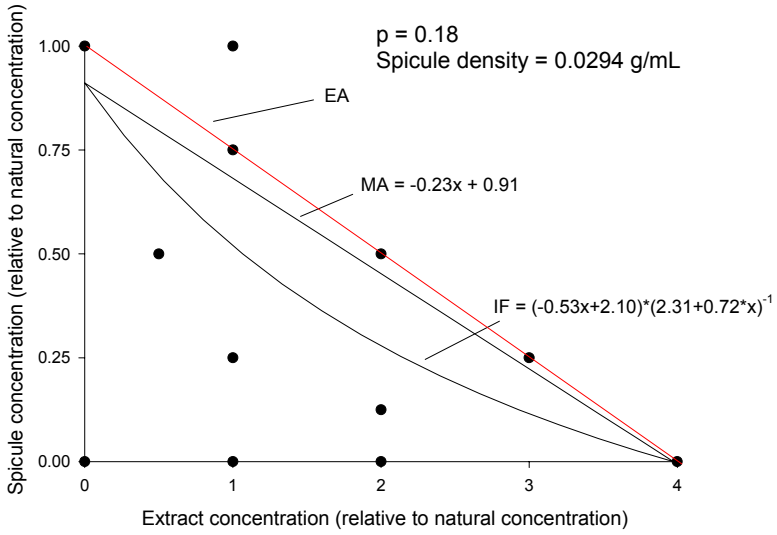


Figure 3: a) 2-D Isobologram for *Cinachyra alloclada*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Cinachyra alloclada*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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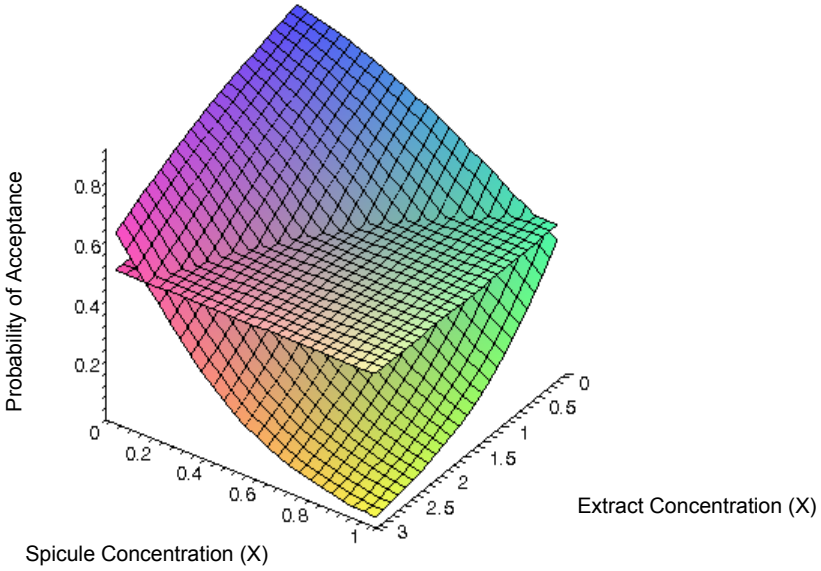
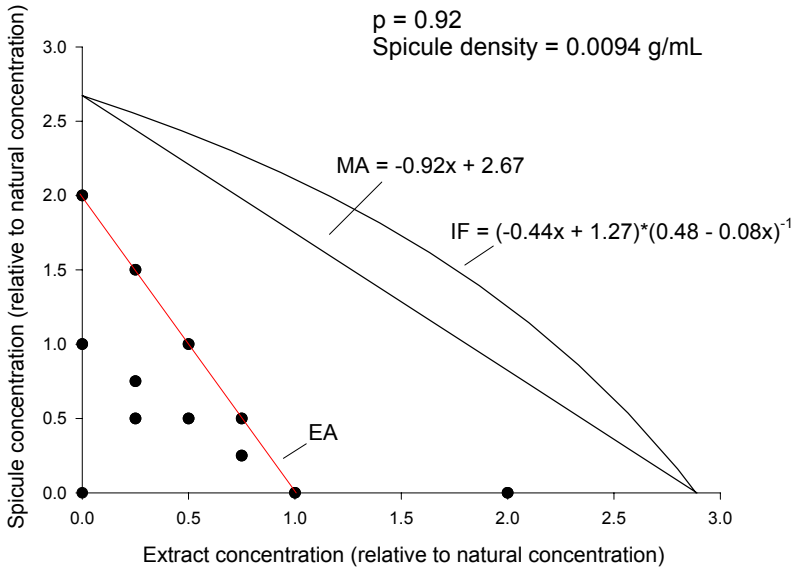


Figure 4: a) 2-D Isobologram for *Cribrochalina vasculum*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Cribrochalina vasculum*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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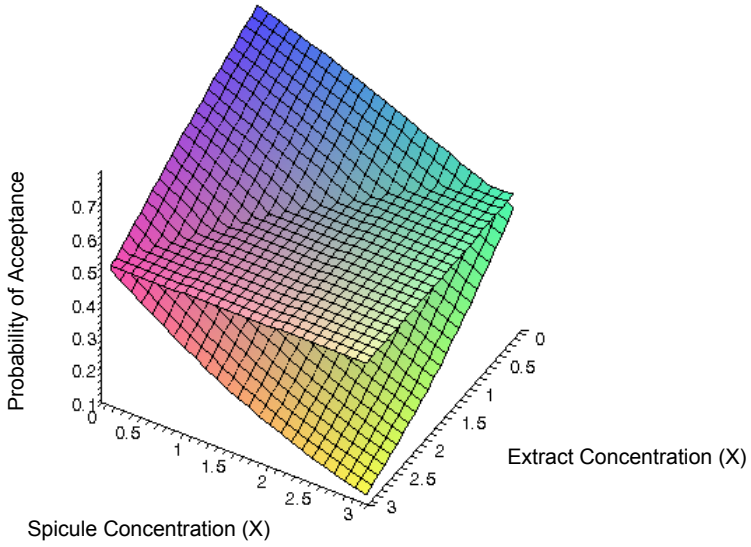
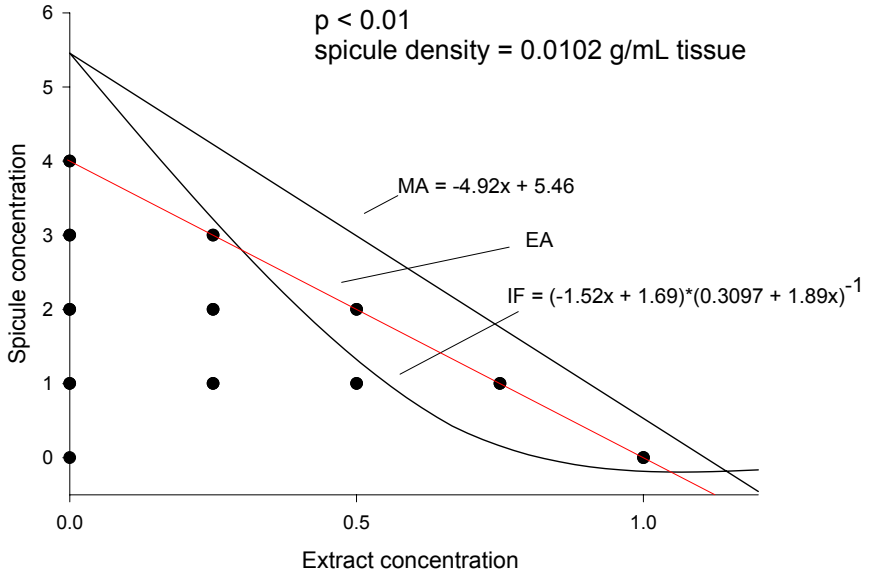


Figure 5: a) 2-D Isobologram for *Niphates digitalis*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Niphates digitalis*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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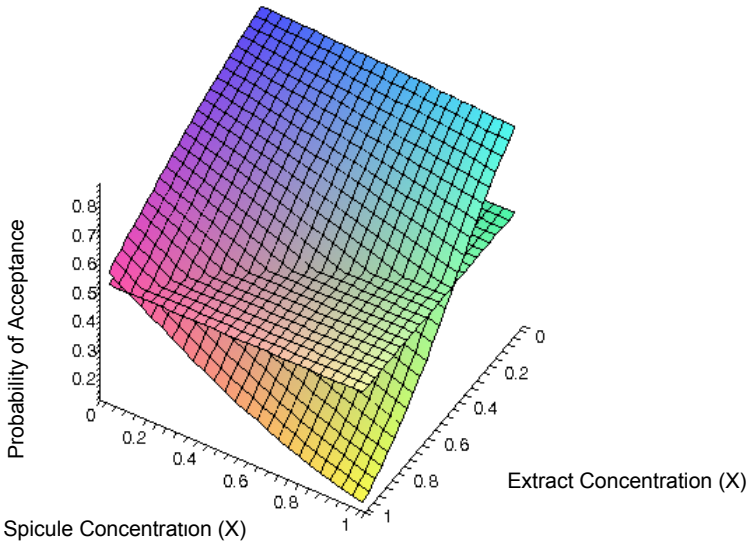
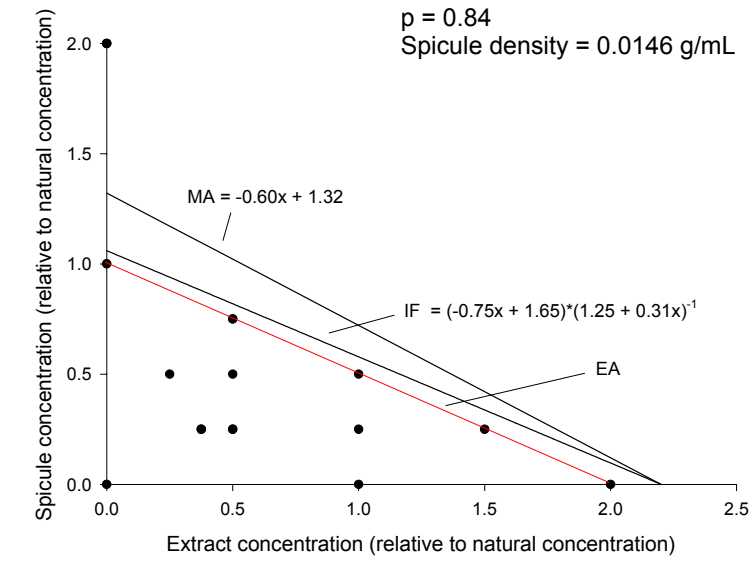


Figure 6: a) 2-D Isobologram for *Rhaphidophlus juniperinus*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Rhaphidophlus juniperinus*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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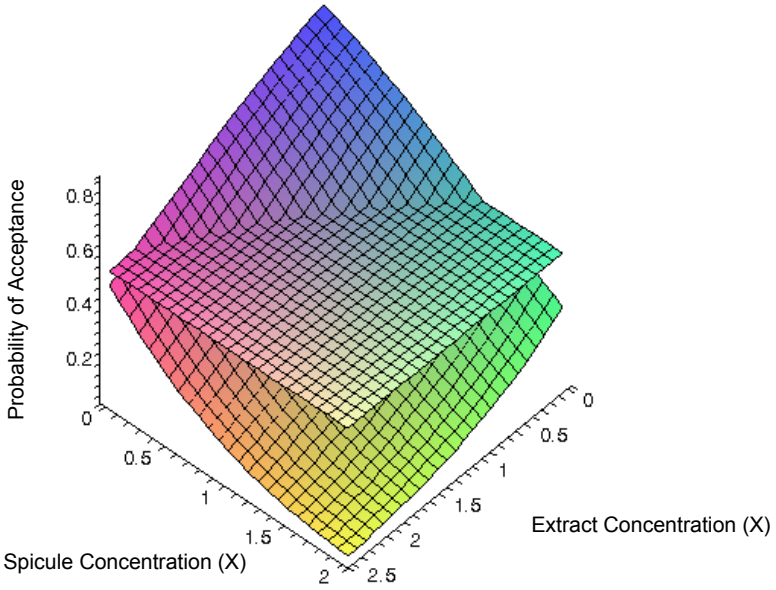
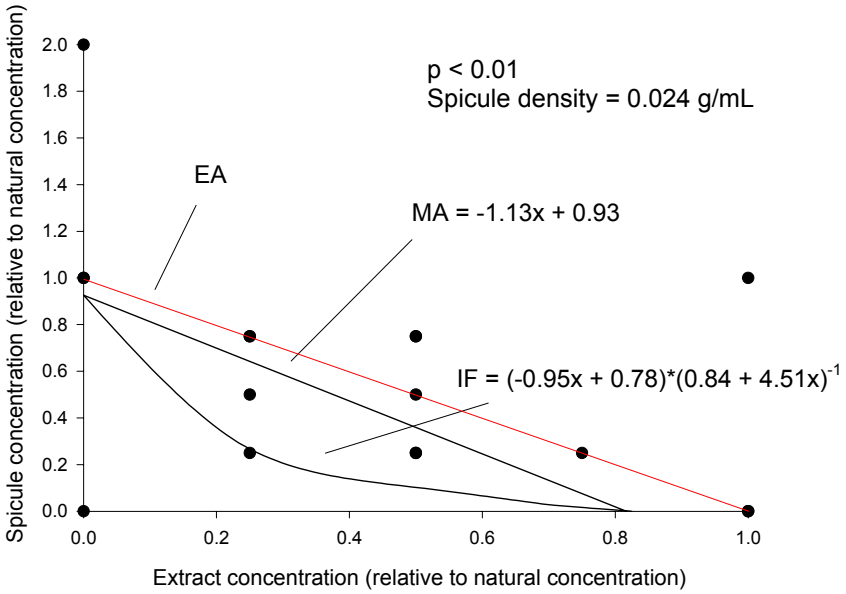


Figure 7: a) 2-D Isobologram for *Tedania ignis*. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Tedania ignis*. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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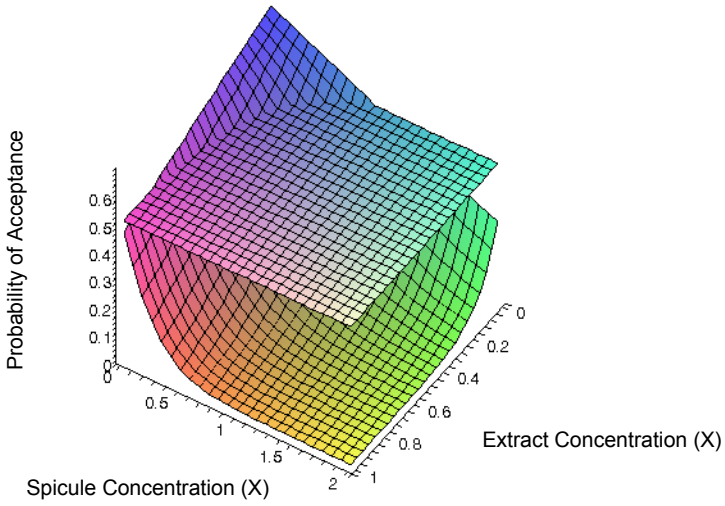
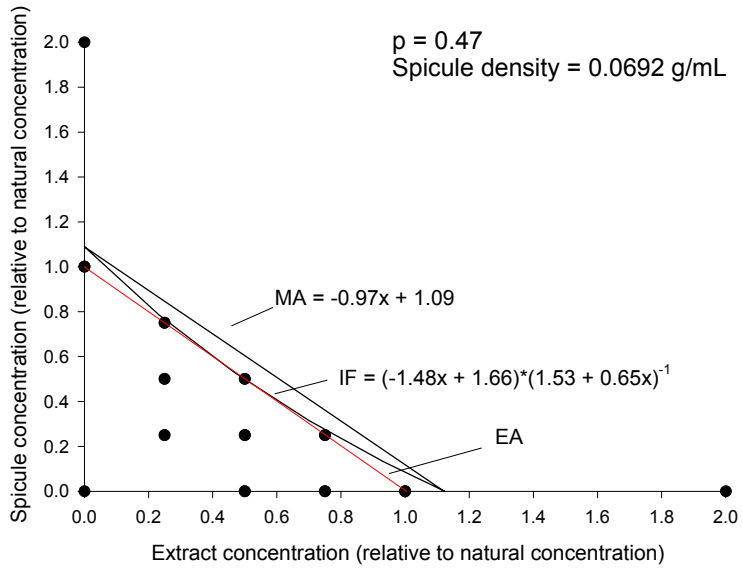


Figure 8: a) 2-D Isobologram for *Xestospongia muta*, Florida Keys sample. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Xestospongia muta*, Florida Keys sample. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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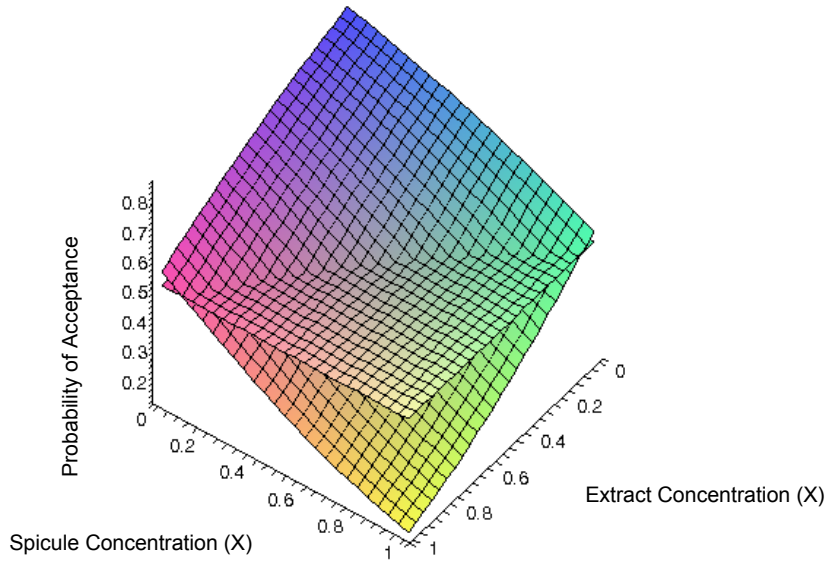
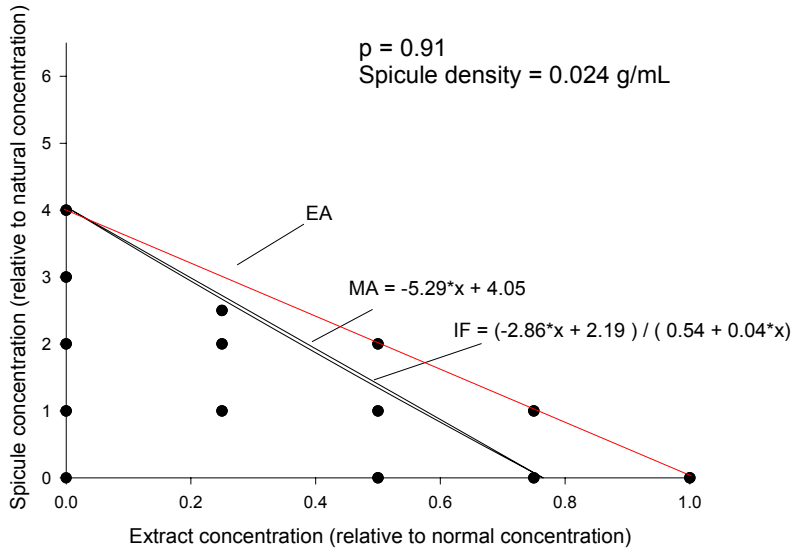


Figure 10: a) 2-D Isobologram for *Xestospongia muta*, Bahamas hard morphology. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Xestospongia muta*, Bahamas hard morphology. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

a)



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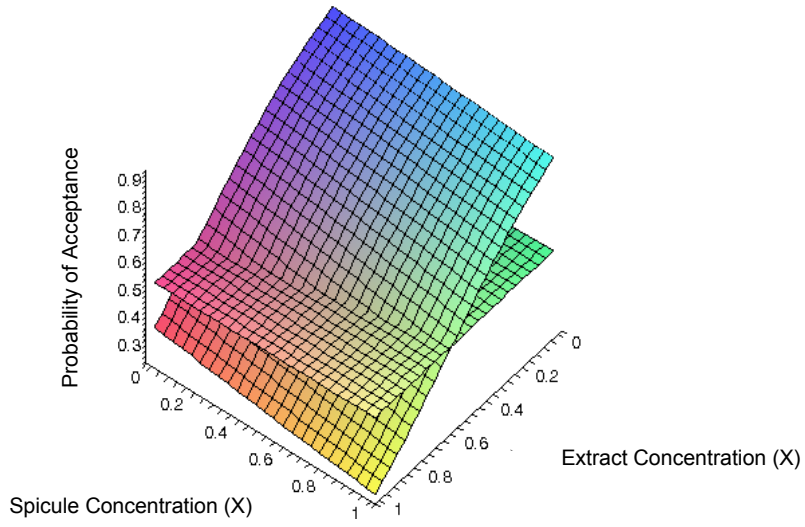

Figure 9: a) 2-D Isobologram for *Xestospongia muta*, Bahamas soft morphology. EA = Experimental additive line, MA = model derived additive line, IF = interaction function. Data points are combination assays conducted. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution. b) 3-D Isobologram for *Xestospongia muta*, Bahamas soft morphology. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

DISCUSSION

Synergistic interactions between crude extracts and spicules were discovered for four Caribbean sponges against a generalist fish predator, *Thalassoma bifasciatum*: *Agelas clathrodes*, *Cinachyra alloclada*, *Rhaphidophlus juniperinus*, and a Florida Keys sample of *Xestospongia muta*. Synergy in *C. alloclada*, *R. juniperinus*, and *X. muta* was detected by using approximate natural concentrations of crude extracts and spicules. Extracts of *A. clathrodes* were deterrent, but combination assays required unnatural reductions in extract concentrations and increases in spicule concentrations to show the synergistic effect. Contrary to the findings of Chanas and Pawlik (1995), spicules from *Cribrochalina vasculum* and two samples of *X. muta* were deterrent at natural concentrations. Spicules from *T. ignis* and *C. alloclada* were deterrent at near-natural concentrations. Sponges with higher spicule densities required less manipulation of their spicule concentrations in laboratory assays to achieve ED_{50} levels, but neither synergy nor chemical defense was correlated with spicule density. These findings indicate that for some sponges, structural elements may serve to enhance chemical defenses against consumers.

Chanas and Pawlik (1995) conducted laboratory and field assays with spicules from 8 sponge species, and found that none of the spicule samples deterred feeding. Chanas and Pawlik (1996) also found that spiculated spongin skeleton was palatable in similar assays, even though spicules embedded in the spongin fibers were oriented in their natural conformation, and that spicules were deterrent when incorporated into artificial foods at reduced protein concentrations. Burns and Ilan (2003) found that spicules from 4 Red Sea sponges and 2 Caribbean sponges were deterrent in feeding assays with the Red Sea wrasse *Thalassoma klunzingeri*, but that spicules from *C. vasculum* were not deterrent. The findings presented here may differ from these studies for two reasons. First, both Chanas and Pawlik (1995) and Burns and Ilan (2003)

bleached small (10 mL) volumetric equivalents of sponge tissue for spicule collection. In preliminary trials conducted in the present study, five 10 mL volumetric equivalents of *A. clathrodes* tissue were bleached and treated following the protocol described in Chanas and Pawlik (1995), and significant variations were found between the dry weights of spicules collected from each equivalent. This may have been due to an unequal distribution of spicules throughout the sponge tissue, possibly in relation to the position of the sponge relative to current or wave action in the field. The bleaching of larger amounts of tissue (200 mL) to determine spicule density provided a mean spicule amount for each assay that allowed for this problem to be avoided. As discussed by both Chanas and Pawlik (1995) and Burns and Ilan (2003), while the spicule arrangement in the tissue of different sponges varies, spicule orientation is haphazard when food pellets are created using this assay technique. However, because Chanas and Pawlik (1996) found no feeding deterrence even when spicules were offered in their natural orientation, this variable should not have influenced the difference in results.

Secondly, *Xestospongia muta* samples from South Abaco, Bahamas were observed during this study to have notably different morphologies, and spicule densities were found to vary on this account. "Hard" and "soft" *Xestospongia* morphologies were first described in the Great Barrier Reef sponge *X. testudinaria* by Fromont (1988), who found that the hard form had complete tracts of spicules encased by spongin fibers, while the soft form had spicule tract junctions bound by spongin fiber development. The *X. muta* samples used by Chanas and Pawlik (1995) could have been of a softer morphology than the two hard forms (the Florida Keys and hard Bahamas samples) used here, and therefore may have not contained sufficient spicule densities to be deterrent. Chemical defenses in *X. muta* can vary significantly based on geographic location (Chanas and Pawlik 1997), but although the Florida Keys *X. muta* sample

exhibited a synergistic interaction while the Bahamas samples did not, all of the *X. muta* samples yielded crude extracts with comparable palatability. Length measurements of 10 spicules from each of the three samples using a ocular micrometer on a compound microscope at 400x were also not significantly different (ANOVA, $p = 0.97$). The variation in interaction type among the *X. muta* samples could be due to a difference in the mixture of metabolites present in each of the samples. The Florida Keys sample could have a chemical component subject to synergy that is not present in the other mixtures.

The advantage of synergistic interactions between chemical components, between chemical and structural components, or between structural components is considerable, since less energy can be designated to defense and these components could be effective against a wider range of competitors. Some evidence is available regarding how interactions between these characteristics may elevate levels of defense. Berenbaum and Neal (1985) found that a terrestrial plant secondary metabolite became significantly more toxic when a second, nontoxic compound was produced that acted as a competitive inhibitor of microsomal mixed function oxidases against predatory insects. In the study done by Hay et al (1994), the proposed synergistic relationship between the algal secondary metabolites and $CaCO_3$ was changed when a lower quality test food was substituted, suggesting that nutritional value was also interacting to affect herbivory.

The mechanism(s) by which secondary metabolites and spicules interact is still unclear. Spicules might abrade digestive surfaces, thus facilitating the intake of secondary metabolites. Synergisms could then be dependent on the type of spicules present, in the event that spicules of a particular size or shape are more effective in causing abrasion. However, because the feeding assay used in the present study was based on an initial acceptance or rejection behavior,

deterrence levels of combination pellets may also have been affected because the inclusion of spicules could have increased handling time of food items. If this was the case, crude extracts of some sponges in the presence of spicules may not be palatable, but the ease with which a fish can swallow a food pellet not containing spicules may allow them to tolerate the presence of secondary metabolites in order to feed. Spicules are not retained in the guts of turtles (Meylan 1988), fish (Randall and Hartman 1968) or invertebrates (Birenheide et al. 1993) and do not appear to cause long-term problems. In the present study, assay fish also did not seem to be affected in cases where spicules were consumed. Chanas and Pawlik (1996) showed that spicules deterred predation if nutritional value of food items is sufficiently lowered. Again, the rapid acceptance or rejection of assay pellets was probably due to either variation in taste, texture, or digestive surface abrasion because the fish could not visually distinguish between control and treatment pellets. Spicules are inert, as opposed to $CaCO_3$ sclerites or granules that may alter the pH of acidic guts, and therefore do not act as an inorganic chemical defense (see Hay et al. 1994; Hay 1996b).

Most experimental evidence indicates that the primary function of structural components in sponges and other organisms such as gorgonians and ascidians is skeletal support. Koehl (1982) described the mechanical design of spicule-reinforced connective tissue and how it enhances tissue rigidity. Spicules increase in density in response to wave force and to sponge tissue damage (Palumbi 1986; McDonald et al. 2002). However, it is unknown if spicules in some sponges are used specifically to augment chemical defense, or if increases in defense are only a side product of structural reinforcement. Walters and Pawlik (in review) found that chemically undefended sponges heal wounds at faster rates than those that are chemically defended, and proposed that energy can be allocated either to faster healing and growth rates or

chemical defenses with slower growth rates. They also studied the material created by sponges to initially cover the wounds and found it to be interlaced with spicules, which may be attributable to spicules being used to add stability to the afflicted area. It may be difficult to distinguish whether spicule densities reflect structural support relative to flow fields or defensive interactions with chemical components. Flow rates at the sites where sponge samples were collected for this study were not measured. One way to look at these relationships more closely would be to collect samples of synergistic sponges from multiple areas, measure in situ flow rates, and compare chemical defenses and spicule densities with combination feeding assay results.

Coley et al. (1985) proposed an evolutionary correlation in terrestrial plants between resource availability and levels of antiherbivore defense. When nutrients are limiting in the environment, plants with slower growth rates are favored (Grime 1979; Chapin 1980), and they can invest substantial amounts of energy into defenses while minimizing nutrient loss. Ample nutrient amounts contribute to faster growth, since photosynthesis and respiration rates can increase and plants can tolerate rapid leaf turnover in order to maintain high levels of energy intake. Defensive compounds may also be selected for based on their construction costs and metabolic effort to maintain their concentrations (Feeny 1976); slower growth favors high construction costs but low maintenance, with the opposite observed in faster growing plants. The fact that coexisting reef sponges vary in both levels of chemical defense and the presence or absence of synergisms implies that resource availability is probably not the primary factor governing variation in sponge defensive mechanisms. Spongivorous fishes have a considerable affect on reef sponge distribution and abundance, which likely confines several sponge species to cryptic refugia or mangrove habitats (Pawlik 1997). If faster growth rates are needed to persist

in the absence of strong chemical defenses, synergisms between secondary metabolites and spicules in sponges with intermediate levels of defense could reduce the amount of energy needed for growth and reproduction.

Components that have evolved for certain functions or as nonfunctioning structures, and later become utilized for additional purposes, can be called exaptations (Gould and Vrba 1982; Arnold 1994) because they have become fit by reason of their form, rather than through natural selection. An addition exaptation is one in which a trait has initial functional advantages, and a new use becomes added to the first (Arnold 1994). A genus of neotropical vines, *Dalechampia* (Euphorbiaceae) has several defensive traits that appear to be examples of addition exaptations, including bracts that function both as a floral advertisement system and as protection of staminate and pistillate flowers, and resins evolved from being rewards for pollinators to defending ovaries, seeds, leaves, and shoot tips (Armbruster 1997). The dual role of spicules as support for the sponge skeleton and as an enhancement of chemical defenses may be an example of an addition exaptation, in which some sponge species are able to gain defensive advantages through the use of a component designed by evolution for an entirely different purpose. This could explain why laboratory bioassays using spicules in isolation are in most cases not deterrent at natural concentrations by themselves, but combination assays can show the synergistic effect. The source of secondary metabolite production within sponges is still unknown, but the independent evolution of chemical defenses and spicules might have occurred if chemical defenses are a product of symbiotic bacteria instead of the sponge itself.

Benefits of statistical procedures

The statistical procedure used in this study combined two different analytical approaches to create a novel way of examining potential synergisms. The isobolographic model is a successful method within pharmacology and is useful because of its flexibility in data analysis and lack of assumptions about interaction mechanisms (Nelson and Kursar 1999) as well as graphic format and visual interpretation (Berenbaum 1977). Therefore, it serves as an alternative to additive or multiplicative designs that introduce complications or limitations in data analysis (Billick and Case 1994). The GENMOD is used throughout statistics, and can analyze a wide range of categorical data sets depending on the distribution type selected.

Using these two methods in concert has considerable potential for other ecological investigations into potential interactions within organisms. The assay procedure based on acceptance or rejection of food items to selected predators can be applied to artificial foods incorporating components from any prey organism in any concentration. The use of GENMOD allows for any assay response to be included, instead of being limited to the inclusion of ED_{50} values with the isobolograph alone. *Thalassoma bifasciatum* provides consistent feeding assay results when assays are replicated, and can accurately give ED_{50} values from which to gauge the concentrations used in combination assays. Similar efforts based on studies that found no defensive role for structural elements using animals such as ascidians (Lindquist et al. 1992) or gorgonians (O'Neal and Pawlik 2002) could reveal interactions that were previously overlooked.

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BIOGRAPHICAL SKETCH

Adam C. Jones was born on May 23rd, 1980, in Concord, Massachusetts and grew up in Groton, MA. In 2002, he graduated *magna cum laude* from Providence College with a Bachelor of Science degree in biology and a minor in history. During his undergraduate career, Adam participated in an NSF Research Experiences for Undergraduates program at Shoals Marine Laboratory through Cornell University. He became interested in working with invertebrate ecology during this period, and after graduating, joined the research lab of Dr. Joseph Pawlik at UNCW. Working under Dr. Pawlik, Adam completed a thesis on defensive interactions in Caribbean sponges, the results of which are shown here. In addition, Adam has participated in three research missions at the NURC facility in Key Largo, FL, and joined two two-week chemical ecology research cruises aboard the *R/V Seward Johnson*.